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<p>The central hypothesis we are addressing is that inhibition of mammary carcinogenesis by n-3 polyunsaturated fatty acids (PUFAs) can be accounted for by their inhibitory effect on the cholesterol biosynthesis (mevalonate) pathway. In Task 1, we have shown that the decrease in mammary gland HMG-CoA reductase seen in LDL-R -/- mice compared to +/+ mice fed a 7% n-6 PUFA diet persists when animals are fed a 20% n-6 PUFA diet. Based on these observations, a long-term experiment is underway to determine whether the lower reductase activity in LDL-R -/- compared to +/+ mice will lead to a lower incidence of mammary tumors. In Task 2, we have prepared reagents to create transgenic mice that over-express the LDL-R in the mammary gland. We are also testing the feasibility of using mini-osmotic pumps to deliver mevalonate to the rat mammary gland. Preliminary experiments for Task 3 have shown the feasibility of using normal and neoplastic human breast epithelial cells to investigate the mechanism of growth inhibition by n-3 PUFAs. Our work will provide a basis for understanding the protective effects of n-3 PUFAs and perhaps other dietary factors on breast cancer development and may lead to mechanism-based strategies for the prevention of this disease.</p>			
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Table of Contents

Cover.....	1.
SF 298.....	2.
Foreword.....	3.
Table of Contents.....	4.
Introduction.....	5.
Body.....	5.
Key Research Accomplishments.....	13.
Reportable Outcomes.....	13.
Conclusions.....	14.
References.....	15.
Appendices.....	17.

INTRODUCTION

Consumption of fish oils rich in n-3 polyunsaturated fatty acids (PUFAs) is associated with a decreased risk of breast cancer in women. These fatty acids also inhibit the development of chemically induced, transplanted and spontaneous mammary tumors in rodents. The molecular mechanism of this inhibition, however, has not been elucidated. We have shown that dietary n-3 PUFAs down regulate 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase while increasing the levels of low-density lipoprotein receptors (LDL-R) in rat mammary glands. HMG-CoA reductase catalyzes the formation of mevalonate that is required by mammalian cells for entry into the S phase of the cell cycle and has been postulated to play a role in cancer development. This enzyme is also rate limiting in cholesterol biosynthesis and is down regulated when intracellular cholesterol levels rise. Thus, the induction of LDL-R by n-3 PUFAs that we have observed may mediate the down regulation of HMG-CoA reductase by internalizing cholesterol-rich LDL particles. We hypothesize that inhibition of mammary carcinogenesis by n-3 PUFAs can be accounted for by their inhibitory effect on the mevalonate pathway. The objective of this research is to test this hypothesis in rodent models, and examine whether a similar mechanism could be operative in human breast cancer development.

BODY

Task 1. To determine the effects of dietary n-3 PUFAs on mammary tumorigenesis in LDL-R +/+ and LDL-R -/- mice (months 1-24).

The first objective of Task 1 was to produce mammary tumors in LDL-R +/+ and LDL-R -/- mice fed diets containing n-3 or n-6 PUFAs. Since writing the grant application, we completed a series of experiments in which we investigated the regulation of mevalonate synthesis in LDL-R -/- mice fed n-3 or n-6 PUFAs (1). Our results showed that dietary PUFAs

and deletion of the LDL-R had independent effects on hepatic and mammary gland HMG-CoA reductase and serum lipids and we observed a significant diet-gene interaction. The effects of PUFAs on HMG-CoA reductase in the mammary gland, but not the liver, were mediated by the LDL-R. We also observed that differences in HMG-CoA reductase and serum LDL-cholesterol, high density lipoprotein cholesterol and triglycerides between -/- and +/+ mice were dependent on whether mice were fed n-3 or n-6 PUFA.

In view of the differences in lipid metabolism that we observed in -/- and +/+ mice fed the different diets, we decided to make a few changes in the design of the tumorigenesis experiment of Task 1. We had proposed to use dimethylbenz(a)anthracene (DMBA) as the initiating agent. DMBA is typically given *i.g.* dissolved in cottonseed oil, and its absorption is mediated by bile acids (2, 3). As a lipid soluble compound, DMBA is transported from the liver to target tissues as a component of blood lipoproteins. We reasoned, therefore, that the difference in serum lipids between LDL-R +/+ and -/- mice would likely lead to differences in the pharmacokinetics of DMBA between the 2 types of mice. Thus, we decided that we must use the water soluble initiating agent methylnitrosourea (MNU) instead of DMBA to prevent the potentially confounding effect which a difference in carcinogen uptake and delivery between groups might have on initiation.

MNU initiates mammary tumorigenesis in mice (4-6), but a potential problem can be the development of lymphomas and ovarian tumors before mammary tumors have had the time to develop (7, 8). For this reason, mice are usually treated with hormones in mammary tumorigenesis experiments to reduce the latency and promote the growth of mammary tumors (5, 6, 8). Indeed, the background strain of the LDL-R -/- mice, C57Bl, is poorly susceptible to chemically-induced carcinogenesis (8), and we felt it would be necessary to treat these animals in some way to promote mammary tumor development. We chose, therefore, to treat all of the

mice with the synthetic hormone medroxyprogesterone acetate (MPA) that has previously been shown to promote mammary tumorigenesis in the mouse (5, 6).

Work we performed to investigate the effects of n-3 and n-6 PUFAs feeding on mammary HMG-CoA reductase activity in LDL-R $-/-$ versus wildtype mice was done using a 7% fat diet (9). However, as we indicated in the grant application, Ip et al (10) have shown that diets must contain at least 4% linoleic acid to ensure adequate levels of this essential fatty acid for maximal mammary tumor growth. While the 7% safflower oil diet we used previously contains adequate linoleic acid, the 7% fat menhaden oil diet would be deficient, and may, therefore, artificially limit mammary tumor growth independent of any specific inhibitory effects of the individual PUFAs. To ensure adequate levels of linoleic acid in any future mammary tumorigenesis studies conducted, we have reformulated the test (n-3) and control (n-6) diets as follows: the 20% fat n-3 diet – 12% menhaden oil, 6% safflower oil, 2% soybean oil; the 20% fat n-6 diet – 18% safflower oil, 2% soybean oil.

In view of these changes from the diets we previously used, we decided it was necessary to perform a preliminary experiment to ensure that the decrease in mammary HMG-CoA reductase activity seen in LDL-R $-/-$ mice compared to wildtype mice fed the 7% n-6 PUFA diet persisted when animals were fed the 20% n-6 PUFA diet. Briefly, 4 LDL-R $-/-$ and 4 LDL-R $+/+$ mice were fed the 20% fat n-6 diet for one week. Animals were sacrificed by CO₂ asphyxiation and microsomes were isolated from mammary and liver tissues. Both mammary and hepatic HMG-CoA reductase activity levels were significantly lower in LDL-R $-/-$ animals versus wildtype (see Figs 1a and 1b), and were, indeed, comparable to those we observed in mice fed the 7% n-6 diet (9).

In view of the issues discussed above, we decided it would be prudent to carry out a preliminary experiment to determine the feasibility of the full experiment described in Task 1.

We decided to focus on the hypothesis that lower the HMG-CoA reductase activity in LDL-R -/- mice compared to +/+ mice will lead to a lower mammary tumor incidence when the mice are fed diets rich in n-6 PUFAs (Fig. 1).

Thirty female LDL-R knockout mice and 30 C57Bl wildtype controls were purchased from Jackson Labs at 5 weeks of age and acclimatized on an AIN-93G standard diet for one week. At 6 weeks of age animals were injected s.c. with 40 mg of slow-release medroxyprogesterone acetate (MPA) (Depo-Provera®) in the interscapular area. At 8 weeks of age, animals received a single dose of 50mg/kg MNU. One week post-MNU all animals were switched to the 20% n-6 diet for the duration of the experiment. Animals were weighed biweekly and palpated for the presence of mammary tumors weekly. Animals were given fresh food biweekly, and consumption per cage was recorded.

Throughout the study there was no significant difference in food consumption or weights between the two groups (see Figs 2a and 2b). Both LDL-R -/- and wildtype groups gained weight progressively for the first three months. Approximately one month post-initiation, some animals unexpectedly began “barbering”, a process of excessive grooming that leads at first to superficial hair loss, but if continued aggressively, to eventual skin damage and tissue destruction in the area affected. By ~2.5 months post-MNU, we sacrificed some animals that had significant damage to the skin and underlying connective tissue, or that had lost weight. Steps were taken to minimize barbering activity & damage – barbered animals were separated to prevent possible barbering by the dominant female in the cage, and animals with skin lesions were treated with varitone to prevent infection, and to lessen damage by reducing irritation in the area. Sacrificed animals were necropsied, and samples of mammary, liver, spleen, kidney, and ovaries were taken for histopathology. Several mice had grossly enlarged spleens, and all had marked anaemia. Because of the barbering activity, we decided to terminate the experiment at 6 months post-initiation. At

this time, all mice were killed and samples of mammary gland, ovaries, liver, kidney, and spleen were placed in 10% buffered formalin for paraffin embedding. Samples of serum were prepared from blood, and frozen at -80°C. No palpable, or grossly visible mammary tumors were evident in either group, although small, circumscribed and sometimes colored areas were grossly visible in the inguinal mammary fat pad of a number of mice. We are currently in the process of preparing slides from tissue samples collected for histopathological examination. Our ability to proceed with Task 1 will depend on the outcome of this preliminary experiment.

Task 2 To determine whether transgenic mice that overexpress the LDL-R in the mammary gland develop fewer tumors than wild-type controls.

We have postponed extensive work on the primary objective of Task 2 pending the outcome of Task 1. We have, however, acquired and prepared the functional expression vector containing the MMTV-LTR promoter/enhancer and the LDL-R cDNA, and we are ready to begin preparation of the transgene construct.

In view of the potential problems associated with the mouse model outlined in Task 1, we have begun to explore other approaches to Task 2. We may be able to make transgenic mice in a strain that is considerably more susceptible to mammary tumorigenesis than the C57Bl mice that we were constrained to use in Task 1. It is also possible that we can make transgenic rats that we know reproducibly give a high yield of mammary tumors. We are investigating these possibilities with our Transgenic Facility. We have, however, been investigating an alternative approach to help us achieve our primary objective – to establish definitively whether mevalonate plays a role in mammary carcinogenesis.

In the rat mammary tumorigenesis model, we would make use of mini-osmotic pumps to deliver mevalonate to the mammary glands. Mevalonate will down-regulate HMG-CoA reductase while at the same time allow cells to make all of the down-stream mevalonate-derived metabolites. This model will be equally as useful for us as the transgenic LDL-R mouse and will avoid the problems associated with the mouse being resistant to mammary carcinogenesis. The experimental design would be as follows. 120 female Sprague Dawley (SD) rats, age 6 weeks, will be acclimatized for 8 days on AIN 93G. At 50 days of age, all animals will be given a single injection of MNU, which reproducibly yields greater than 80% mammary tumor incidence in SD rats within 6 months. Animals will then be randomized to 2 groups and fed either the n-3 diet, or the n-6 diet. One week post-MNU, under ketamine/xylazine anaesthesia, animals on both diets will be further randomized to 2 subgroups, with half of the animals on each diet receiving an *s.c.* implanted mini-osmotic pump containing 1mg/uL mevalonate, and the other half receiving a pump containing isotonic saline (controls). At monthly intervals, spent pumps will be replaced with fresh pumps containing either test (mevalonate), or control (saline) solutions, respectively. At approximately 6 months post-MNU, animals will be sacrificed. Histopathology will be performed on all tumors, and HMG-CoA reductase activity will be assessed for tumors and representative normal mammary tissue.

This model has several advantages compared to experiments in mice: the rat model is well established to produce a high incidence of mammary tumors (>80% in our hands) with a short latency (<26 weeks), rats do not require hormonal stimulation in order to generate mammary tumors and MNU has a relatively low toxicity in rats compared to mice.

To assess the feasibility of performing the above experiment, we have determined the ability of mini-osmotic pumps to deliver a physiological dose of mevalonate to the mammary glands. Effective delivery of mevalonate was assessed by measuring feedback inhibition of

HMG-CoA reductase. Briefly, 10 female SD rats, age 12 weeks, were randomized to two groups, and surgically implanted *s.c.* with a 200 uL mini-osmotic pump (flow rate of 0.25uL/hour, and pumping 'life' 28 days) containing either 1mg/ul mevalonate, or isotonic saline. Rats were sacrificed approximately two weeks after pump implants, and HMG-CoA reductase activity was assessed in rat mammary gland and liver. Results indicated no significant difference in mammary or liver HMG-CoA reductase activity between groups (see Figs 3a & 3b), indicating insufficient delivery of mevalonate to mammary cells by 200 uL pumps.

This experiment brought to light a problem with our assay – hepatic and mammary HMG-CoA reductase activity levels were not as high as expected for SD rats. Through the use of control samples, it was determined that the loss of activity resulted during the isolation of microsomes, rather than during the assay of enzyme activity. Several probable causes for the loss of activity have been explored, including the use of halothane as an anaesthetic prior to killing, the effects of freezing tissues prior to microsome isolation, rather than using fresh tissue, and the effects of variations in pH of the isolation buffer. All appeared to demonstrate important effects and, as a result, a number of changes have been made to our protocol. These include the isolation of microsomes from fresh tissue only, the use of CO₂ asphyxia for killing, and the purchase of a new pH-metre/electrode sensitive to Tris-buffers. A considerable amount of time has been spent identifying the source of these various problems, and optimizing analytical procedures. However, although the changes described above to our tissue preparation protocol have yielded some improvements in our ability to accurately measure HMG-CoA reductase activity in rat tissues, we do not currently have consistent, acceptable reproducibility of these measurements. In an effort to overcome this problem, we are preparing anti-HMG-CoA reductase antibodies reactive with the catalytically active domain of the enzyme. This will aid us in two ways. First, it will allow us to measure HMG-CoA reductase protein levels, thereby quantifying the effects of

treatments on enzyme levels, and providing supportive data for our measurements of HMG-CoA reductase activity. Second, it will allow us to visualize the intact enzyme (100 kDa, and 200 kDa) and its cleavage products (57 kDa) during the isolation protocol.

In view of the results with the first preliminary experiment using mini-osmotic pumps that indicated insufficient mevalonate was being delivered to the rats, we decided to do a second experiment with larger pumps. This experiment follows the same protocol as described above, but 2 mL mini-osmotic pumps are used in place of 200 uL pumps. These pumps deliver 2.5 mg mevalonate per hour (based on a mean flow rate of 2.5 uL/hr) to the rat, resulting in a total daily delivery of 64mg. This level is approximately equivalent the daily whole body mevalonate synthesized endogenously by these rats. We are in the process of completing this experiment.

Task 3 To determine the extent to which growth inhibition by n-3 PUFAs is reversed by mevalonate in normal and neoplastic breast epithelial cells.

In the grant application, we had planned to begin Task 3 in the second year. We decided, however, to begin to characterize the cell cultures and the effects of PUFAs on their growth during this first project period. The first objective of task 3 was to establish growth conditions of all cell lines and concentration-response profiles for the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). We have determined seeding density profiles for the normal human breast epithelial cell line MCF-10 and the human breast cancer cell lines MCF-7 (estrogen dependent) and MDA-MB-231 (estrogen independent) (Figs 4a – 4c). Preliminary experiments on the treatment of these cells with EPA or DHA for 24 h indicated a reduction in cell proliferation only at high concentrations of the fatty acids (> 100uM) (Figs 5a – 5c). It is likely that these effects were due to toxicity. Exposure of MB231 cells for longer periods of time at concentrations < 100uM showed a significant time-dependent reduction in growth rate that is

probably due to inhibition rather than toxicity (Figs 6a, 6b). These studies are very preliminary, but do indicate the feasibility of the experiments proposed in Task 3.

KEY RESEARCH ACCOMPLISHMENTS

- The decrease in mammary gland HMG-CoA reductase seen in LDL-R -/- mice compared to +/+ mice fed a 7% n-6 PUFA diet was shown to persist when the animals were fed a 20% n-6 PUFA diet.
- Based on the above results, a long-term carcinogenesis experiment is underway to determine whether the lower HMG-CoA reductase activity in LDL-R -/- mice compared to +/+ mice will lead to a lower mammary tumor incidence.
- The feasibility of using mini-osmotic pumps to deliver mevalonate to the rat mammary gland has been investigated. Our results indicate that a delivery rate of 0.25 mg/h is insufficient to down-regulate mammary gland HMG-CoA reductase and a rate of 2.5 mg/h is currently under test.
- Preliminary experiments have shown the feasibility of using cultures of normal and neoplastic human breast epithelial cells to investigate the mechanism of growth inhibition by n-3 PUFAs.

REPORTABLE OUTCOMES

None at this time

CONCLUSIONS

In the first year of this grant, we have concentrated our efforts on performing experiments that lay the groundwork for the Tasks we proposed. For Task 1, we have shown that the decrease in mammary gland HMG-CoA reductase seen in LDL-R $-/-$ mice compared to $+/+$ mice fed a 7% n-6 PUFA diet persists when the animals are fed a 20% n-6 diet. These observations allowed us to perform a preliminary long-term carcinogenesis experiment to determine whether the lower reductase activity in LDL-R $-/-$ mice compared to $+/+$ mice will lead to a lower incidence of mammary tumors. We await the results. Although we have prepared reagents to create LDL-R transgenic mice as we proposed in Task 2, preliminary results from Task 1 suggest that the mouse model may be too resistant to mammary carcinogenesis to meet our needs. We have, therefore, initiated experiments to test an alternative approach in which mevalonate will be delivered to the mammary gland of rats via mini-osmotic pumps. Mevalonate will down-regulate HMG-CoA reductase while at the same time allow cells to make all of the down-stream mevalonate-derived metabolites. This model will be equally as useful for us as the transgenic LDL-R mouse and will avoid the difficulties of the latter. The model we will use will depend on the outcome of our experiment in Task 1. Preliminary experiments for Task 3 have shown the feasibility of using normal and neoplastic human breast epithelial cells to investigate the mechanism of growth inhibition by n-3 PUFAs.

The successful completion of our experiments will provide a basis for understanding the protective effects of n-3 PUFAs and, perhaps, other dietary factors on breast cancer development and may lead to mechanism-based strategies for the prevention of the disease.

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APPENDIX**Figures**

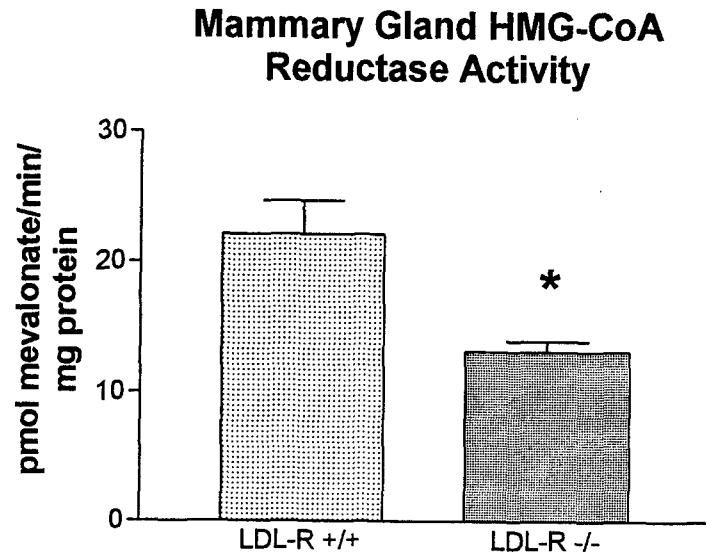


Figure 1a Mammary HMG-CoA reductase activity in LDL-R-/- versus LDL-R +/+ mice fed a 20% fat diet rich in n-6 PUFAs. * p<0.02.

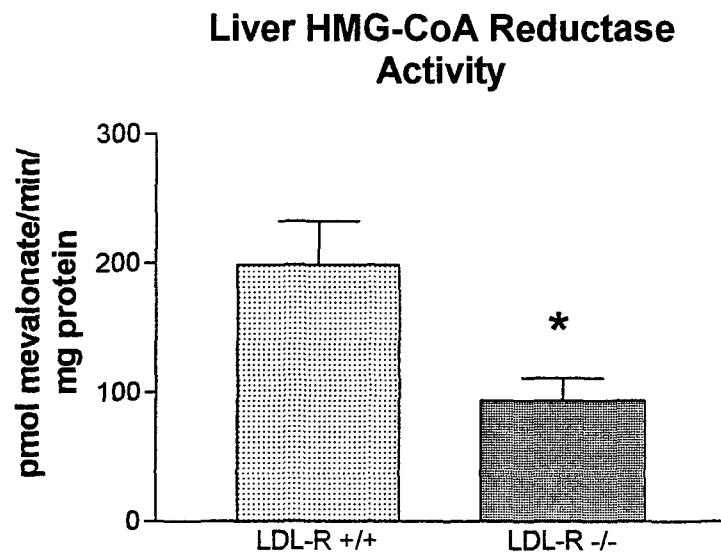


Figure 1b Liver HMG-CoA reductase activity in LDL-R-/- versus LDL-R +/+ mice fed a 20% fat diet rich in n-6 PUFAs. *p<0.05.

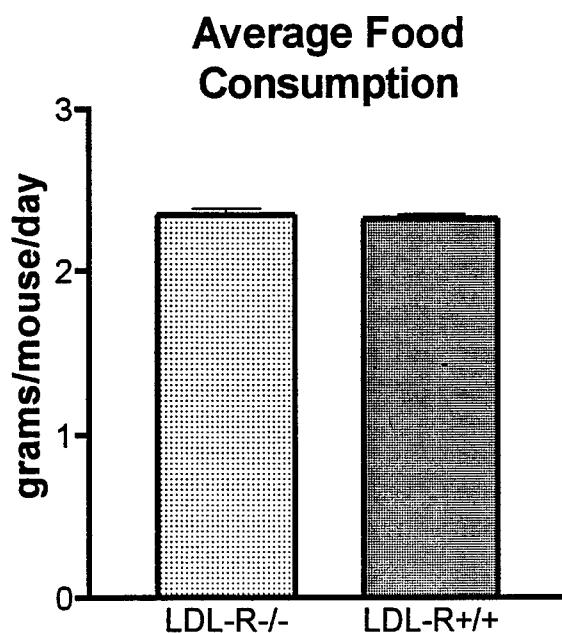


Figure 2a Average daily consumption of n-6 diet per mouse.

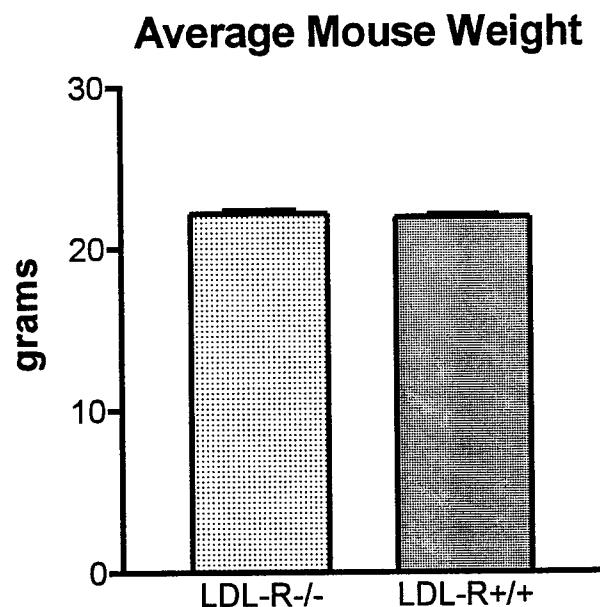


Figure 2b Average mouse weight by group (measured twice weekly throughout study).

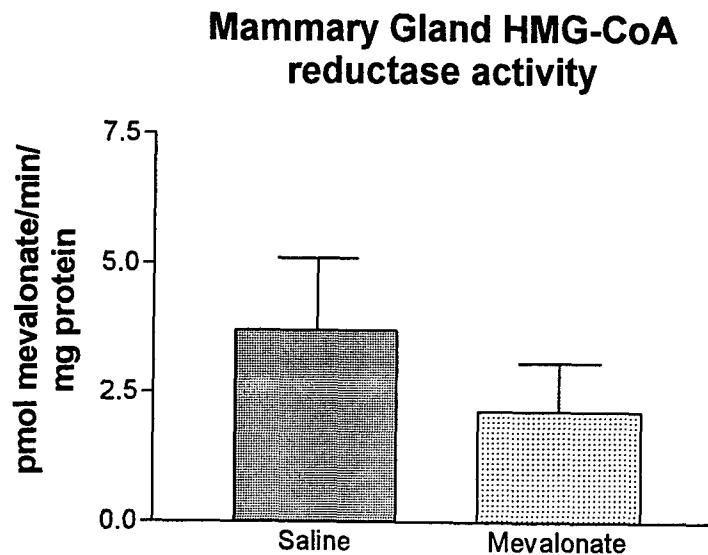


Figure 3a Mammary HMG-CoA reductase activity in rats implanted s.c. with a mini-osmotic pump delivering mevalonate (0.25 mg/h) or isotonic saline (0.25uL/h).

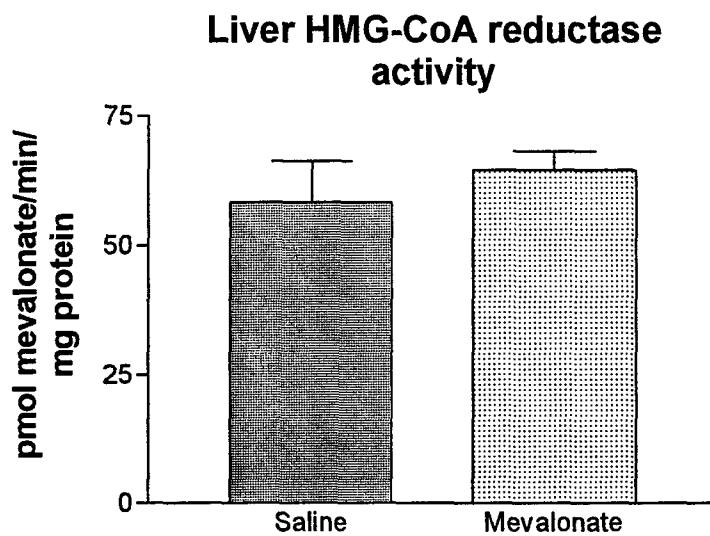


Figure 3b Liver HMG-CoA reductase activity in rats implanted s.c. with a mini-osmotic pump delivering mevalonate (0.25 mg/h) or isotonic saline (0.25uL/h).

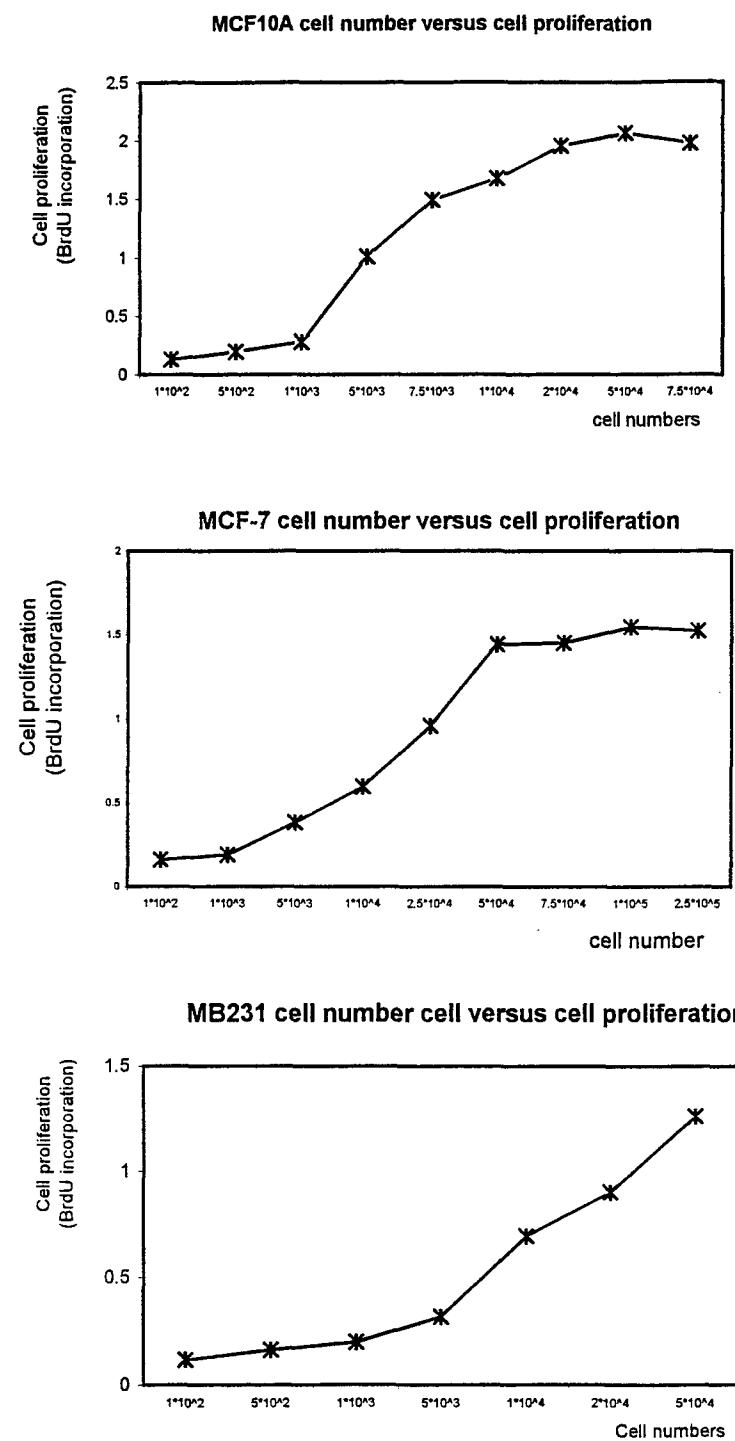


Figure 4 Growth of (a) MCF10A, (b) MCF7, (c) MB231 cells at various seeding densities

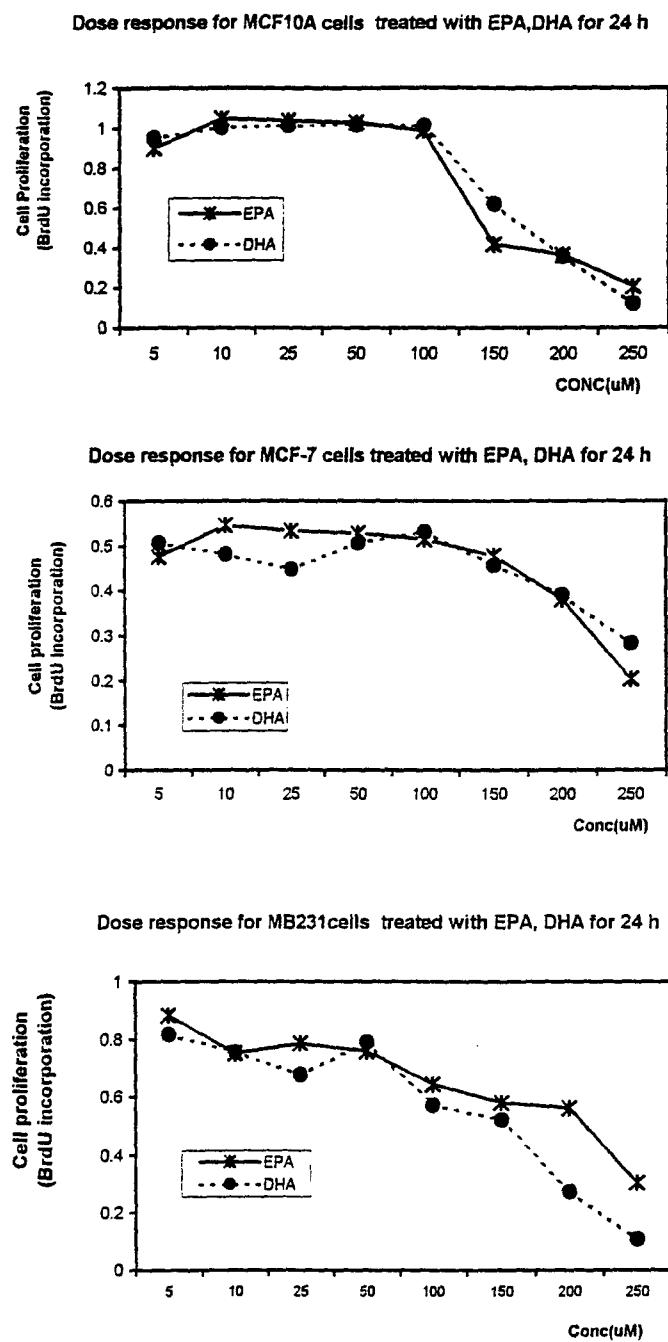
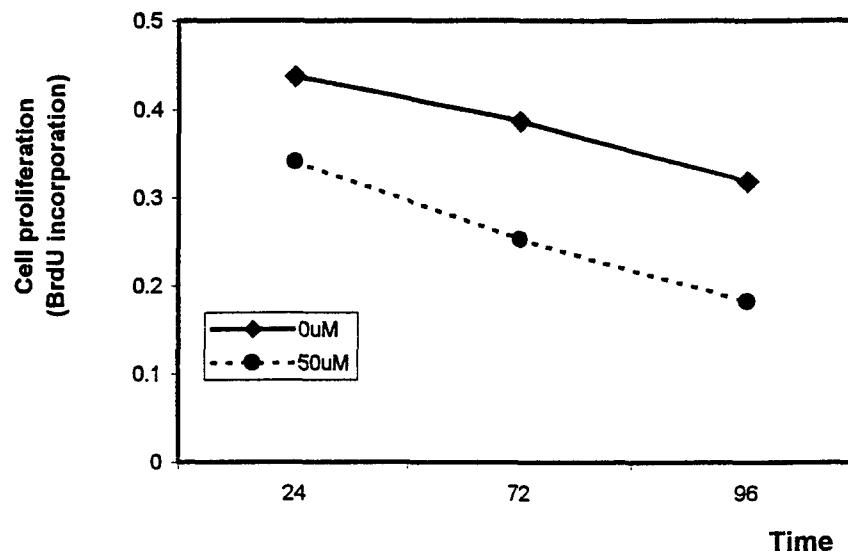
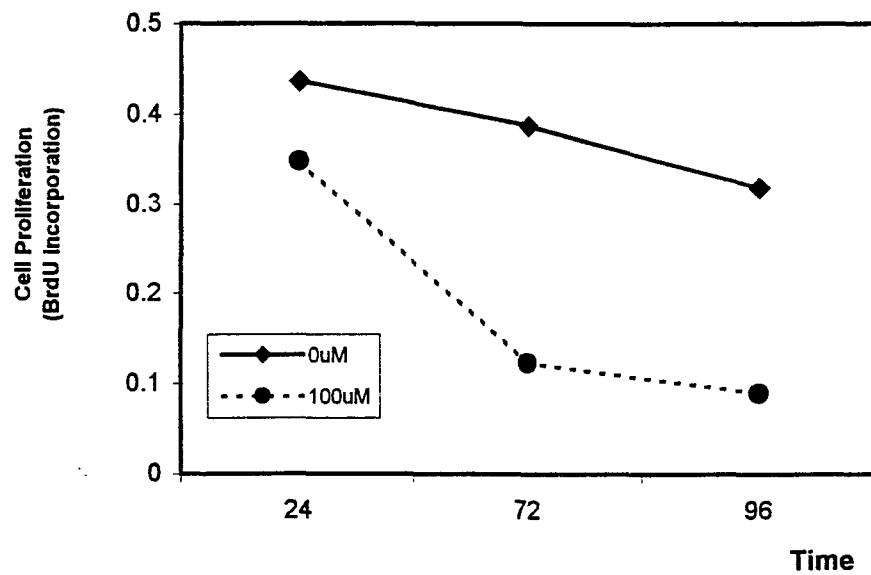


Figure 5 Growth of (a) MCF10A, (b) MCF7, (c) MB231 cells versus concentration of EPA or DHA in the culture medium

Time response for MB231 cells treated with 50 uM DHA

a

Time response for MB231 cells treated with 100uM DHA

b

Figure 6 Growth of MB231 cells in (a) 50 uM, (b) 100 uM DHA for various periods of time